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Inhibition of adenosine kinase attenuates interleukin-1- and lipopolysaccharide-induced alterations in articular cartilage metabolism

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Summary

Objective: To investigate the effect of adenosine kinase inhibition on interleukin (IL)-1β- and lipopolysaccharide (LPS)-induced cartilage damage.

Design: Articular cartilage was obtained from the metacarpophalangeal joints of 10 young adult horses. Following a stabilization period, weighed cartilage explants were exposed to IL-1β (10 ng/ml) or LPS (50 μg/ml) to induce cartilage degradation. To test the potential protective effects of adenosine, these explants were simultaneously exposed to adenosine (100 μM), the adenosine kinase inhibitor 5′iodotubercidin (ITU, 1 μM) or to both adenosine and ITU. After 72 h in culture, conditioned medium was collected for evaluation of glycosaminoglycan (GAG), nitric oxide (NO), prostaglandin E_2 (PGE_2) and matrix metalloproteinase (MMP)-3 release.

Results: IL-1β and LPS stimulated significant release of GAG, NO, PGE_2 and MMP-3. Incubation with ITU significantly inhibited both IL-1β- and LPS-induced GAG release, but did not alter MMP-3 production. Exposure to ITU also reduced IL-1β-induced PGE_2 release and LPS-induced NO production. Direct adenosine supplementation did not attenuate the effects of IL-1β or LPS, and the addition of adenosine or ITU in the absence of IL-1β or LPS did not have any detectable effect on cartilage metabolism in this model.

Conclusions: The adenosine kinase inhibitor ITU attenuated experimentally induced cartilage damage in an *in vitro* cartilage explant model. Release of adenosine from chondrocytes may play a role in the cellular response to tissue damage in arthritic conditions and modulation of these pathways in the joint may have potential for treatment of arthropathies.

Key words: Adenosine, Articular cartilage, Cartilage damage, Interleukin-1β.

Introduction

In healthy articular cartilage, resident chondrocytes are responsible for maintenance of the extensive extracellular matrix that surrounds them, providing for low-friction, painless joint movement[1,2]. Chondrocytes are capable of responding to physiological alterations to preserve matrix integrity; however, in disease states, regulation of chondrocyte metabolism is lost, resulting in depletion of extracellular matrix and abnormal biomechanical function[2,3]. The failure to maintain tissue homeostasis in osteoarthritis (OA) is ultimately reflected in gross pathologic lesions, mechanical dysfunction and progressive loss of comfortable joint movement. However, the pathologic triggers that disrupt joint equilibrium in OA remain poorly defined.

Current experimental evidence suggests that degradation of cartilage matrix in diseased joints is mediated through complex interactions between a number of different factors including inflammatory cytokines, matrix degrading enzymes, nitric oxide (NO), oxygen derived free radicals and prostaglandins[2–4]. In addition, a growing literature suggests that extracellular purine nucleotides and nucleosides also participate in this complex process[5]. The purine base adenosine is released from stressed or damaged cells including articular chondrocytes[6]. Adenosine can function as an extracellular signaling molecule, and has been reported to attenuate inflammatory responses in a variety of *in vivo* models and body systems[7–11]. Extracellular adenosine interacts with P1 cell surface receptors that have been classified into four subtypes, A_1, A_{2A}, A_{2B} and A_3[12]. These purine receptors are found on many cell types throughout the body, and articular chondrocytes express the A_{2A} and A_{2B} adenosine receptor genes[13]. Moreover, because adenosine is a metabolic breakdown product of adenosine triphosphate, some researchers have speculated that adenosine receptors may have evolved in part to protect tissues from various harmful stimuli[9]. Considering the documented anti-inflammatory role of adenosine receptor activation in other body systems, it is plausible that adenosine plays an essential role in synovial joint homeostasis[5]. In fact, there are a number of studies confirming that adenosine deaminase concentrations are elevated in naturally occurring inflammatory joint conditions, and this

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elevation would be expected to reduce the concentration of potentially protective extracellular adenosine present in affected joints[14–16]. In addition, in rodent models of inflammatory arthritis, activation of adenosine receptors has been shown to inhibit inflammation[17–19], while adenosine agonists suppress synoviocyte collagenase gene expression in culture[20].

There is also specific evidence that receptor-mediated adenosine pathways may be important regulators of chondrocyte activity. Adenosine and specific A_2 receptor agonists stimulate intracellular cyclic adenosine monophosphate accumulation and inhibit inflammatory mediator-induced release of NO from articular chondrocytes[21,22]. In addition, chondrocytes can be stimulated to regulate the concentration of adenosine available at the cell surface, indicating the potential of this mediator as an autocrine regulatory factor in cartilage[6]. Furthermore, endogenously released adenosine appears to regulate cartilage matrix integrity through activation of cell surface receptors[23].

In vivo, extracellular adenosine is rapidly depleted either by enzymatic conversion to inosine via extracellular adenosine deaminases or by transporation of extracellular adenosine into the cell where it is phosphorylated by intracellular adenosine kinases[24]. The relatively short half-life of adenosine in biological systems limits the therapeutic value of direct adenosine administration[25], but adenosine kinase inhibitors can be used experimentally to preserve endogenously synthesized adenosine. The resulting increase in intracellular adenosine levels promotes passive facilitated diffusion out of the cells, and a subsequent increase in extracellular adenosine concentrations[26,27]. Adenosine kinase inhibitors are also being investigated for their ability to increase extracellular adenosine concentrations *in vivo* providing therapeutic potential as anti-inflammatory agents[28]. Several adenosine kinase inhibitors, including 5′iodotubericidin (ITU), have been found to be efficacious both *in vitro* and *in vivo*[28], and adenosine kinase inhibitors have been used to curtail adjuvant-induced arthritis in rats[29]. In differentiated chondrocyte cultures, ITU was capable of stimulating a sustained increase in extracellular adenosine concentrations[6].

The objective of the current study was to determine whether adenosine kinase inhibition is capable of protecting cartilage against the deleterious actions of interleukin (IL)-1 and bacterial lipopolysaccharide (LPS). We compared IL-1 and LPS-induced glycosaminoglycan (GAG), NO and prostaglandin E2 (PGE_2) release from cartilage explant cultures in the presence and absence of supplemental adenosine and the adenosine kinase inhibitor, ITU.

Materials and methods

MATERIALS

Commercial immunoassays for PGE_2 and matrix metalloproteinase (MMP)-3 were purchased from R&D Systems (Minneapolis, MN, USA). All standard chemicals and reagents including adenosine, LPS, ITU and recombinant human IL-1β (rhIL-1β) were acquired from Sigma-RBI (St. Louis, MO, USA) while the nutrient supplement ITS^+ was purchased from Collaborative Biomedical Products (Bedford, MA, USA). The Dulbecco's Modified Eagle's Medium (DMEM) F-12 tissue culture medium, and amphotericin B were purchased from Gibco-BRL (Grand Island, NY, USA). Gentamicin sulfate was obtained from Schering-Plough Animal Health Corp (Madison, NJ, USA).

TISSUE COLLECTION AND CULTURE

Full-thickness articular cartilage was aseptically harvested from the metacarpophalangeal and metatarsophalangeal joints of 10 equine cadavers. The horses were between 1 and 6 years of age and had died or were euthanatized for reasons unrelated to the musculoskeletal system. In addition, none of the horses had clinically detectable joint disease. The dissections were performed as described elsewhere[30], and during the dissection, cartilage was visually inspected to ensure that only grossly normal, non-calcified cartilage was collected for the study. Cartilage from the metacarpophalangeal and metatarsophalangeal joints of individual horses was pooled for that horse. However, cartilage from different horses was maintained and studied independently.

Following the dissection, harvested cartilage was rinsed twice with sterile phosphate buffered saline (pH 7.3) and subsequently cultured in sterile serum-free maintenance medium consisting of DMEM F-12 containing a universal nutrient supplement ITS^+, 2.5 μg of amphotericin B/ml, 50 μg of ascorbic acid/ml, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 50 μg of gentamicin sulfate/ml. Cartilage slices obtained during the dissection were diced into approximately $5 \times 5 \times 3$ mm pieces of cartilage, and 75–105 mg of wet weight cartilage was placed into sterile polystyrene culture tubes. During the following 48-h stabilization period, the pre-weighed explants were incubated in 2 ml of maintenance medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

ADDITION OF MEDIATORS TO EXPLANT CULTURES

After the initial 48-h stabilization period, maintenance medium was discarded and the explants were incubated in 1.5 ml of experimental treatment media for 72 h (treatment period). All experimental media were supplemented with nutrient and antimicrobial additives as previously described. For this study, time zero was defined as the time that incubation with treatment medium was initiated after the 48-h stabilization period. During the treatment period, groups of explants were incubated in control media or in the presence of IL-1 or LPS at concentrations that have elicited maximal inflammatory responses in previous experiments using this model [30,31] (Table I). Explants in the induced matrix

Table I
Effects of an adenosine kinase inhibitor on IL-1β- and LPS-induced cartilage degradation. Articular cartilage explants were cultured for 72 h in medium containing one of the following inflammatory mediators and treatment additive combinations

Descriptor	Inflammatory mediator	Treatment additive
Control	None	None
IL-1-alone	rhIL-1β 10 ng/ml	None
IL-1 + ITU	rhIL-1β 10 ng/ml	ITU 1 μM
IL-1 + adenosine	rhIL-1β 10 ng/ml	Adenosine 100 μM
IL-1 + ITU and adenosine	rhIL-1β 10 ng/ml	ITU 1 μM + adenosine 100 μM
LPS-alone	LPS 50 μg/ml	None
LPS + ITU	LPS 50 μg/ml	ITU 1 μM
LPS + adenosine	LPS 50 μg/ml	Adenosine 100 μM
LPS + ITU and adenosine	LPS 50 μg/ml	ITU 1 μM + adenosine 100 μM
ITU-alone	None	ITU 1 μM
Adenosine-alone	None	Adenosine 100 μM
ITU + adenosine	None	ITU 1 μM + adenosine 100 μM

degradation categories were exposed to one of the following: 50 μg LPS/ml from *Escherichia coli* serotype 055:B5 ($n = 8$ horses), or 10 ng rhIL-1β/ml ($n = 5$ horses).

To investigate the ability of adenosine or ITU to inhibit induced cartilage degradation, explants exposed to IL-1β or LPS were assigned to one of four groups: explants incubated with 100 μM adenosine, explants incubated with 1 μM ITU, explants incubated with both 100 μM adenosine and 1 μM ITU, and explants exposed to IL-1β-alone or LPS-alone without additional additives. All treatment combinations and controls were established in triplicate for each animal. Standard control explants were cultured in fresh maintenance medium alone. Additional experimental controls included explants cultured with adenosine, ITU or both adenosine and ITU in the absence of LPS and IL-1.

SAMPLE COLLECTION

Tissue culture medium was removed from the explant tubes after 72 h of incubation, and the 1.5 ml volume was aliquoted into four samples for subsequent determination of GAG, NO, PGE_2 and MMP-3 content. The culture medium and the cartilage explants were stored separately at −20°C prior to being assayed. To permit determination of the total GAG content remaining in the cartilage explants following the 72-h incubation period, the explants were digested with activated papain. Papain (0.5 mg/ml) was added to a 0.2 M phosphate buffer (pH 6.5) containing ammonium acetate (0.1 M), ethylenediaminetetraacetic acid (5 mM), and cysteine hydrochloride (5 mM). This mixture was incubated at 60°C for 30 min to activate the enzyme. The explants were then incubated with 1 ml of the activated solution at 60°C for 24 h.

QUANTIFICATION OF GAG RELEASE

Cartilage matrix proteoglycan degradation was estimated by measuring GAG release using a 1,9-dimethymethlene blue metachromatic dye assay modified for use in microtiter plates[32]. Following the 72-h incubation, tissue culture media GAG content was assayed directly, and GAG content remaining in the explants was determined from the papain digested cartilage diluted 1:15 in 0.2 M phosphate buffered saline (pH 6.5). All samples were read in triplicate at a 540 nm wavelength, and shark chondroitin sulfate was used to generate a standard curve. The percent of GAG released from the explants was determined as a percentage of total GAG.

MEASUREMENT OF NO RELEASE

NO release was assessed by measuring nitrite levels in the medium using a spectrophotometric method based on the Griess reaction[33]. Because nitrite is a stable oxidant product of NO, and approximately half the total NO released by chondrocytes will accumulate as nitrite in the culture medium, NO levels in culture medium can be estimated by measuring nitrite concentrations[34]. Sodium nitrite in tissue culture medium (2.5−100 μM) was used to create the standard curve for this assay. Two-hundred microliter aliquots of sample or standard medium was combined with 200 μl of the Griess reagent (1:1 ratio of 0.1% napthylethylenediamine dihydrochloride in distilled water, and 1% sulfanilamine in 5% H_3PO_4), and centrifuged to remove precipitates. After centrifugation, 100 μl aliquots of the samples and standards mixed with the Griess reagent were added in triplicate to a 96-well plate, and the optical density was measured at 570 nm using a microplate reader. Nitrite levels were determined based on the standard curve generated for each plate.

QUANTIFICATION OF PGE_2 RELEASE

PGE_2 concentrations were determined using a commercially available competitive enzyme-linked immunosorbent assay (ELISA) kit. Samples and PGE_2 standards provided with the kit were diluted 100 fold with DMEM F-12 medium, and transferred to a 96-well plate coated with goat anti-mouse monoclonal antibodies. Alkaline phosphatase-labeled PGE_2 and mouse monoclonal anti-PGE_2 antibodies were added to each well for competitive binding with sample or standard PGE_2. After an initial 2-h incubation, wells were washed to remove excess conjugate and unbound sample, and *p*-nitrophenyl phosphate substrate was added for a 1-h incubation while color developed. The optical density of each well was then determined at 405 nm with a correction wavelength of 570 nm. In this assay, color intensity is inversely proportional to PGE_2 concentration, and a standard curve was used to estimate concentrations of PGE_2 in each well.

QUANTIFICATION OF MMP-3 RELEASE

MMP-3 concentrations were determined using a commercially available quantitative sandwich ELISA kit, designed to measure total MMP-3 (pro- and active forms). Samples diluted 1:10 with a buffered protein base and MMP-3 standards were transferred to a 96-well plate coated with a polyclonal mouse antibody against MMP-3. All samples and standards were assayed in duplicate. The plate was incubated for 2 h, washed to remove unbound substances, and polyclonal antibody against MMP-3 conjugated to horseradish peroxidase was added for an additional 2-h incubation. Following another wash, a substrate solution of hydrogen peroxide and tetramethylbenzidine was added, and the plate was incubated in the dark for 30 min. Two molar sulfuric acid was then added as a stop solution, and the optical density was measured at 450 nm with a correction wavelength of 570 nm. A standard curve was used to estimate concentrations of MMP-3 in each well.

STATISTICAL ANALYSIS

All values are expressed as mean ± S.E.M. Statistical analysis was performed using commercial statistical software (SAS PROC GLM, SAS 8.01 TS Level 01M0, 1999−2000, SAS Institutes Inc., Cary, NC). Prior to including data from a given horse in the final statistical analysis, a Student's *t* test was performed for each horse comparing GAG release data from control explants with values from explants exposed to LPS-alone and IL-1-alone to determine if the cartilage from that individual horse had responded appropriately to the stimulation for matrix degradation. A value of $P < 0.05$ was considered significant. If there was no significant difference between control values and LPS-alone or IL-1-alone values for GAG release, data from that horse were not included in the final analysis as it would not be possible to evaluate the ability of adenosine or ITU to prevent inflammatory mediator-induced degradation in those horses.

A blocked analysis of variance (ANOVA) was used to determine significant treatment and horse effects

($P < 0.05$). A Shapiro–Wilk test ($P < 0.05$) was performed to assess normality of the data, and a Levene test for homoscendasticity ($P < 0.05$) was used to determine whether the residual variance was constant across treatments. If the Levene test was significant, then a weighted ANOVA was performed and used in lieu of the original ANOVA.

When the ANOVA or the weighted ANOVA indicated that the model was significant, *post-hoc* means comparisons were performed to determine differences between groups. A Dunnett's test ($P < 0.05$) was performed on the full data set to compare values for control explants with all other explant groups. Another blocked ANOVA followed by a *post-hoc* Dunnett's test analysis was performed on a subset of the data comparing LPS-alone or IL-1-alone values with each of the treatment groups cultured in presence of that inflammatory mediator. Finally, a Tukey test ($P < 0.05$) was used to further evaluate the significance of other pairwise comparisons.

Results

SAMPLE POPULATION

Stimulation of cartilage explants with the inflammatory mediators LPS or IL-1β resulted in an increase in GAG, NO PGE_2 and MMP-3 release. Both LPS and IL-1β induced significant NO release by explants from all 10 horses tested. However, cartilage from two horses did not release appreciable levels of GAG in response to either LPS or IL-1β ($P > 0.05$), and those horses were excluded from subsequent data analyses as specified in the study design. In summary, an appropriate GAG release response to LPS was observed in seven of eight horses tested with LPS, and four of the five horses tested with IL-1β. Explants from three horses were tested in parallel with IL-1β and LPS, and responded to both. Only data from LPS and IL-1 responsive horses were included for evaluation of the response to adenosine and ITU treatment, and in total, cartilage explants from eight different horses between 1 and 4 years of age were included in the final analysis and results.

In the absence of LPS or IL-1β, treatment of explants with either adenosine, the adenosine kinase inhibitor ITU, or with both ITU and adenosine did not have a significant effect on GAG, NO, PGE_2 or MMP-3 release. Data for these control groups are not included separately in the figures due to the absence of a difference from standard control explants incubated in maintenance medium alone.

ITU INHIBITS LPS- AND IL-1β-INDUCED GAG RELEASE

Treatment with ITU alone or in combination with adenosine significantly reduced LPS- and IL-1-induced GAG release (Figs. 1 and 2). Although GAG release was more substantially inhibited by the co-addition of ITU and adenosine than by ITU alone, the difference between these two treatment groups was not statistically significant.

ITU INHIBITS LPS AND IL-1β-INDUCED NO RELEASE

Cartilage cultured in maintenance tissue culture medium alone produced very low levels of NO, and stimulation with LPS or IL-1β resulted in a dramatic increase in NO release. NO release stimulated by LPS (mean 0.59 nmol/mg cartilage ± S.E.M. 0.05) was significantly greater than that observed with IL-1β (mean 0.40 nmol/mg cartilage ± S.E.M. 0.05).

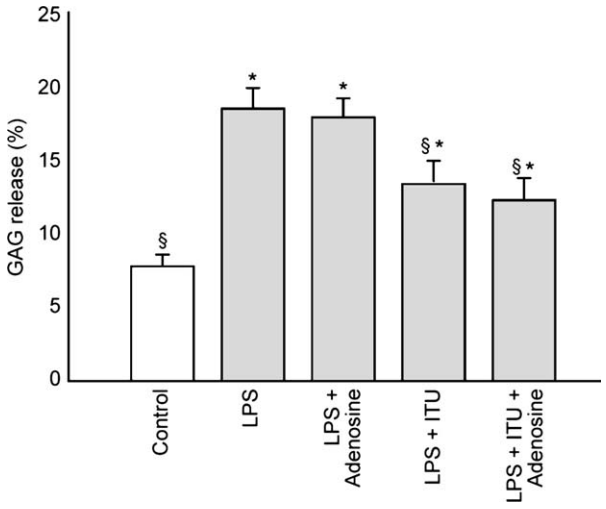

Fig. 1. Percentage of total GAG content released from cartilage into explant culture medium during a 72-h incubation period in maintenance medium alone (control), with LPS (50 μg/ml) alone, or with LPS in combination with adenosine (100 μM), ITU (1 μM), and both adenosine and ITU. Values reported represent the mean results obtained for explants from seven horses; error bars represent the S.E.M. *Significantly ($P < 0.05$) different from values for control explants. §Significantly ($P < 0.05$) different from values for explants incubated with LPS-alone.

Treatment of cartilage explants with ITU alone or in combination with adenosine resulted in a substantial attenuation of LPS-induced NO release (Fig. 3). Explants exposed to LPS and treated with a combination of ITU and adenosine produced less NO than those treated with ITU alone; however, this difference was not statistically significant. Treatment with adenosine alone did not significantly

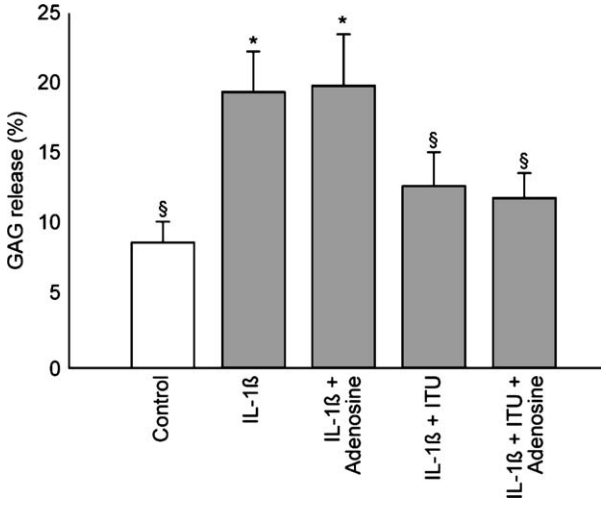

Fig. 2. Percentage of total GAG content released from articular cartilage into explant culture medium during a 72-h incubation period in maintenance medium alone (control), with IL-1β (10 ng/ml) alone, or with IL-1β in combination with adenosine (100 μM), ITU (1 μM), and both adenosine and ITU. Values reported represent the mean results obtained for explants from four horses; error bars represent the S.E.M. *Significantly ($P < 0.05$) different from values for control explants. §Significantly ($P < 0.05$) different from values for explants incubated with IL-1β alone.

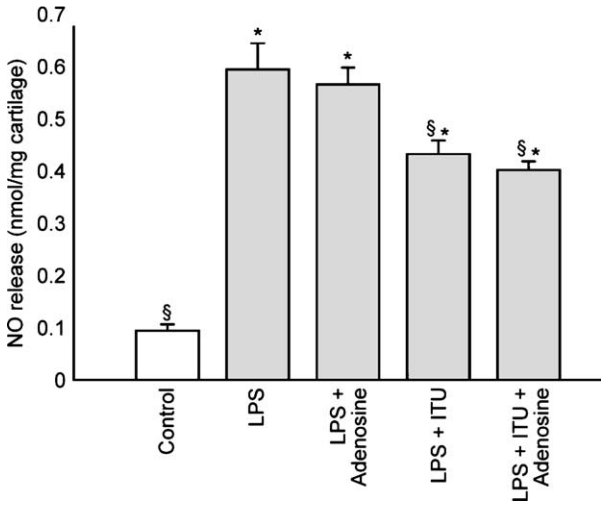

Fig. 3. NO release by cartilage explants cultured for 72 h in maintenance medium alone (control), with LPS (50 μg/ml) alone, or with LPS in combination with adenosine (100 μM), ITU (1 μM), and both adenosine and ITU. NO release was measured indirectly by use of the Griess reaction and is expressed as nanomoles of nitrite per mg of cartilage wet weight. Values reported represent the mean results obtained for explants from seven horses; error bars represent the S.E.M. *Significantly ($P < 0.05$) different from values for control explants. §Significantly ($P < 0.05$) different from values for explants incubated with LPS-alone.

inhibit LPS-induced NO release. Cartilage treated with ITU alone or in combination with adenosine showed a trend towards attenuation of IL-1-induced NO release; however, the suppression of NO production was not statistically significant (Fig. 4). Treatment with adenosine alone did not significantly affect IL-1-induced NO release.

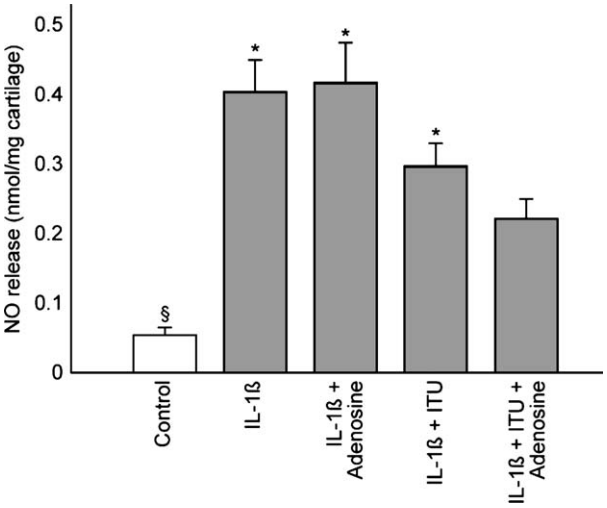

Fig. 4. NO release by cartilage explants cultured for 72 h in maintenance medium alone (control), with IL-1β (10 ng/ml) alone, or with IL-1β in combination with adenosine (100 μM), ITU (1 μM), and both adenosine and ITU. NO release was measured indirectly and is expressed as nanomoles of nitrite per mg of cartilage wet weight. Values reported represent the mean results obtained for explants from four horses; error bars represent the S.E.M. *Significantly ($P < 0.05$) different from values for control explants. §Significantly ($P < 0.05$) different from values for explants incubated with IL-1β alone.

ITU INHIBITS IL-1β- AND LPS-INDUCED PGE_2 RELEASE

Cartilage cultured in maintenance tissue culture medium alone produced low levels of PGE_2, and stimulation with LPS or IL-1β resulted in a substantial increase in PGE_2 release. Levels of PGE_2 detected after a 72-h incubation with LPS-alone (mean 2123 pg/mg cartilage ± S.E.M. 234) were higher than levels detected after stimulation with IL-1β-alone (mean 1270 pg/mg cartilage ± S.E.M. 131). Treatment of cartilage with ITU either alone or in combination with adenosine resulted in a reduction of IL-1-induced PGE_2 release (Fig. 5). Incubation with IL-1β and simultaneous treatment with ITU alone or in combination with adenosine returned PGE_2 production to levels similar to that of control. Treatment of cartilage explants with adenosine and ITU alone or in combination showed a strong trend toward inhibition of LPS-induced PGE_2 release but the values were not statistically significantly different (Fig. 6).

ITU DOES NOT INFLUENCE IL-1β- AND LPS-INDUCED MMP-3 RELEASE

Explants cultured in maintenance tissue culture medium produced low levels of MMP-3, and stimulation with LPS or IL-1β resulted in a substantial increase in MMP-3 release. Concentrations of MMP-3 detected in the medium following incubation with IL-1β alone (mean 9.49 ng/mg ± S.E.M. 25) were higher than levels detected after incubation with LPS-alone (mean 6.97 ng/mg ± S.E.M. 1.19). The addition of adenosine, ITU or a combination of both did not inhibit LPS- or IL-1-induced MMP-3 production (data not shown).

Discussion

There is increasing evidence that adenosine plays a direct role in the pathophysiology and prevention of articular

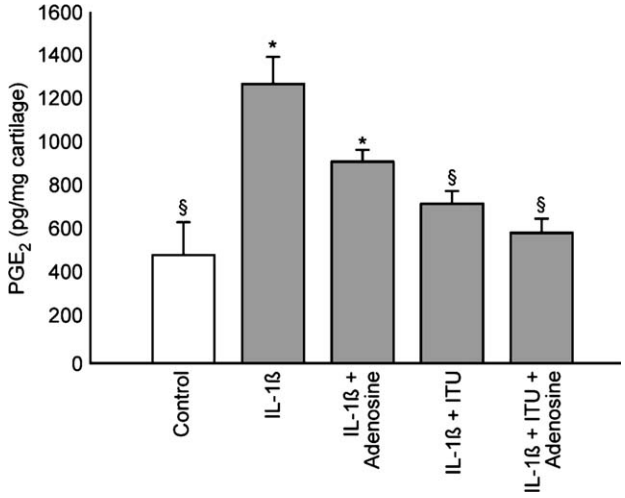

Fig. 5. PGE_2 released from cartilage explants cultured for 72 h in maintenance medium alone (control), with IL-1β (10 ng/ml) alone, or with IL-1β in combination with adenosine (100 μM), ITU (1 μM), and both adenosine and ITU. PGE_2 concentrations were determined using a competitive ELISA kit and are expressed as pg per mg of cartilage wet weight. Values reported represent the mean results obtained for explants from four horses; error bars represent the S.E.M. *Significantly ($P < 0.05$) different from values for control explants. §Significantly ($P < 0.05$) different from values for explants incubated with IL-1β alone.

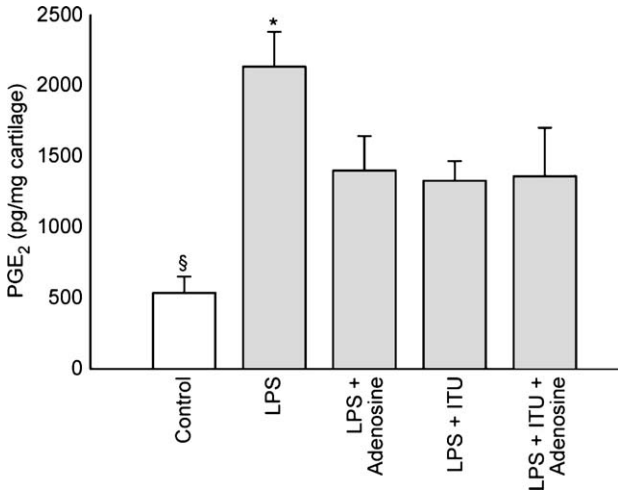

Fig. 6. PGE_2 released from cartilage explants cultured for 72 h in maintenance medium alone (control), with LPS (50 μg/ml) alone, or with LPS in combination with adenosine (100 μM), ITU (1 μM), and both adenosine and ITU. PGE_2 concentrations were determined using a competitive ELISA kit and are expressed as pg per mg of cartilage wet weight. Values reported represent the mean results obtained for explants from four horses; error bars represent the S.E.M. *Significantly ($P < 0.05$) different from values for control explants. §Significantly ($P < 0.05$) different from values for explants incubated with LPS-alone.

cartilage damage[5,21–23], and the study reported here provides support for that theory. IL-1 is an important mediator of cartilage degradation in OA and related inflammatory joint conditions[35], and LPS is thought to be a critical mediator of the destructive processes associated with gram-negative bacterial joint infections[36]. As a result of the catabolic responses consistently observed in culture, both IL-1 and LPS are frequently utilized in experimental models to investigate joint disease, and both mediators worked effectively in this study to stimulate GAG, NO and PGE_2 release.

Several physiological mechanisms can be exploited experimentally to increase extracellular adenosine concentrations. Adenosine kinase inhibition was specifically chosen for the current study based on recent evidence that articular chondrocytes, exposed to the adenosine kinase inhibitor ITU, accumulated extracellular adenosine[6], while use of an adenosine deaminase inhibitor in the same experimental model did not produce detectable extracellular adenosine levels. Furthermore, chondrocytes exposed to a combination of LPS with ITU produced significantly larger quantities of adenosine[6], suggesting that adenosine levels would be substantially elevated for a sustained period in our induced-inflammation model.

In this study, direct adenosine supplementation did not block the deleterious effects of IL-1 or LPS exposure. In chondrocyte monolayer cultures, exogenously supplemented adenosine was depleted from the extracellular pool within minutes[6], with a mean half-life of 1.49 min, suggesting that exogenously supplemented adenosine would be unlikely to effectively diffuse to receptors at the cell surface of chondrocytes within intact matrix. Earlier studies using isolated chondrocytes did document inhibition of LPS-induced NO release with adenosine supplementation alone[21,22]. However, those studies were performed using high-density monolayer chondrocytes cultures where added adenosine would immediately contact cell surface receptors. Furthermore, pre-incubation with adenosine prior to IL-1 or LPS exposure was reported to be necessary for a significant response[22]. In general, the short half-life of adenosine suggests that a source of sustained adenosine release such as an adenosine kinase inhibitor would be necessary to provide anti-inflammatory benefits[9,28], and our results support that observation.

The most plausible interpretation of the results presented in this study is that the responses to ITU detected were due to elevations in extracellular adenosine. Alternative explanations should also be considered including the possibility that ITU was acting through another mechanism. ITU is a potent adenosine kinase inhibitor[37–39], and it has frequently been used in experiments to increase extracellular adenosine levels[6,40,41]. The 1 μM concentration used in this study was selected based on the dose response of articular chondrocytes exposed to ITU in which maximal adenosine release was detected with 1 μM ITU without an adverse impact on cell viability[6]. ITU is generally considered to function specifically as an adenosine kinase inhibitor at this concentration although other potential mechanisms have been documented when the drug is used at higher concentrations[42,43].

Another explanation for the observations reported here is that the anti-inflammatory benefits of ITU are secondary to accumulation of adenosine breakdown products like inosine or other downstream products of adenosine utilization by adenosine deaminase. However, previous work indicates that the direct application of inosine in chondrocyte cultures is not an effective anti-inflammatory additive[23], and inosine challenge does not mimic the effects of adenosine in the lung[44].

In this explant study, suppression of NO release from LPS stimulated explants exposed to ITU or ITU and adenosine confirms previous observations made in monolayer cultures[21,22], and supports the anti-inflammatory benefits of ITU. For the IL-1 stimulated explants we were not able to document a statistically significant reduction in NO release from explants, although there was a strong trend in that direction. Similarly, PGE_2 is routinely measured as an indicator of inflammation, and reduced levels in the explant model used here suggest a suppressed inflammatory response with inhibition of adenosine kinase. In the current study we demonstrated that ITU suppressed PGE_2 production by explants co-incubated with IL-1β. However, while there was a strong trend toward a similar result in the LPS stimulated explants, there was not a significant suppression of PGE_2 production. As observed with NO, the release of PGE_2 was greater with LPS than with IL-1, and this may have influenced the differences in response observed between the two.

There is considerable evidence that degradation of cartilage matrix in OA is mediated by MMPs, including collagenases 1 and 3 (MMP-1, MMP-13) and stromelysin 1 (MMP-3)[45]. Specifically, MMP-3 is involved in the activation of latent collagenase and is regarded as the predominant enzyme involved in the degradation of non-collagen components of cartilage matrix[46]. In this study, production of MMP-3 was evaluated to determine if alterations in MMP-3 were responsible for the observed changes in GAG release. While both LPS and IL-1 exposure did increase total MMP-3 levels, we were not able to document any reduction in MMP-3 levels with ITU exposure despite significantly reducing GAG release in those cultures. Although regulation of adenosine levels has been shown to influence MMP release[20], our results are actually comparable to those reported from a synoviocyte cell

culture model, in which adenosine receptor activation failed to regulate IL-1 stimulated MMP-3 production[20]. Future studies should investigate the role of collagenases in ITU- and adenosine-mediated cartilage protective effects.

In the current study, GAG release was measured as an indicator of proteoglycan degradation. The addition of ITU with or without supplemental adenosine resulted in a significant reduction in the percentage of total GAG released from the explants exposed to both IL-β and LPS, with the most dramatic responses detected in IL-β stimulated cultures. This provides evidence that manipulation of extracellular adenosine levels may not only have immediate anti-inflammatory benefits, inhibiting PGE_2 and NO release, but may also have the potential to minimize associated long-term cartilage damage. While we did not completely reverse the effects of IL-1 and LPS in this model, these results do suggest that manipulation of adenosine levels may be a valuable therapeutic avenue to explore *in vivo*.

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